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RESEARCH ARTICLE

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Sulfite-reducing clostridia in the sediment of a high mountain lake (Laguna Grande, Gredos, Spain) as indicators of fecal pollution

Summary We studied the vertical distribution of sulfite-reducing clostridia in the sediment of a Spanish high-mountain lagoon (Laguna Grande de Gredos, central Spain), with optimal sediment characteristics (temperature < 20°C) to maintain spores without growing. This allowed us to assess the original numbers of sulfite-reducing clostridia endospores settled, without postdepositional growing. Sulfite-reducing clostridia are normal inhabitants of the intestinal microbiota of humans and other mammals. These microorganisms may form endospores, which allow the bacteria to survive in almost any habitat, either terrestrial or aquatic, waiting for favorable conditions for growth. Sulfite-reducing clostridia could be suitable indicators of past human pollution because they have a great longevity in natural habitats and they cannot multiply at temperatures below 20°C or in the presence of O_2. We found a great increase in the numbers of clostridia (expressed as colony-forming units per gram [CFU/g] of dry weight of sediment) since the 1970s, which reflects the rise of human pressure caused by the practice of outdoor activities.Clostridia CFU/g rose dramatically after the faulty operation of the depuration system of a mountain refuge built close to the lagoon. We compared the vertical distribution of clostridia CFU/g from Laguna Grande sediments with those from a neighbor lagoon (Laguna Cimera), which showed less tourist pressure and no direct disposal of sewage. Finally, we agree with the usefulness of the numbers of sulfite-reducing clostridia as indicators of past pollution.

Key words *Clostridium* endospores · Sulfite-reducing clostridia · Lake sediments · High-mountain lakes · Fecal indicators

Introduction

The history of every lake, as well as many events happened in its catchment, is stored in its sedimentary record in physicochemical or biological vestiges (paleoindicators). This information is filed in layers, which can be dated by radiometric techniques. Sulfite-reducing clostridia are common components of the intestinal microbiota of humans and other mammals. These microorganisms may form endospores, which cannot reproduce at temperatures lower than 20°C or in the presence of oxygen. The extreme stability of clostridial spores to various environmental conditions, including those present at the bottom of lakes, suggests that bacterial cell concentrations recovered from sediments might reflect the amount of spores settled and be an indicator of fecal pollution at the time of settling [10, 16].

The presence or absence of sulfite-reducing clostridia is commonly used to evaluate the sanitary quality of water, sediment and animal samples [1, 4, 5, 13, 19]. They have been proposed to fix microbiological standards in the revision of the EU Directive on drinking water, e.g. absence of sulfite (SO_3^{2-})-reducing clostridia in 100 ml [8]. Under unfavorable conditions, clostridia, like other endospore-forming bacteria (e.g. the genera *Bacillus*, *Thermoactinomyces*), can survive for a long time as resting cells in lake water and sediments [3, 9, 12, 15, 20]. Clostridia has also been used as a paleolimnological indicator of pollution by human effluents in rivers [2, 14, 17] and in benthic coastal marine environments [6, E. Martínez-Manzanares (1989) Ph.D.Thesis Univ Málaga].

In this article we describe the vertical distribution of sulfite-reducing clostridia at different sediment levels of Laguna Grande de Gredos, Spain, and at two levels of a nearby lagoon (Laguna Cimera), which shows less human pressure. We discuss the relationship between the number of clostridia endospores found and the changes of sewage pollution in the catchment area.

High-mountain oligotrophic aquatic ecosystems, such as that in Laguna Grande, were well preserved in the past because they are distant from both inhabited areas and roads. Since the 1960s, however, mountain landscapes have undergone increasing human impact. Laguna Grande is an example of this trend; at least 32,742 people visited the catchment of the lagoon in 1993. Next to the lagoon, a mountain refuge was built in 1972 that has hosted around 5000 visitors per year. Until 1995, its sewage was treated in a small pond located between the refuge and the lagoon. Since 1995, the refuge has had a depuration system. The resultant effluent flows towards the lagoon through drainage pipes surrounded by gravel and sand in a ditch.

From summer 1996 to August 1997, several circumstances such as a big fissure in one of the tanks, low winter temperatures, and temporary lack of power, led to flaws in the depuration system. The depuration system was repaired in August 1997, but during the repair work 6000 liters of waste water were disposed without any treatment. Some part of that waste water ended in the lagoon. Evidence for environmental problems in Laguna Grande were earlier described by Granados and Toro [11].

Materials and methods

Study area Laguna Grande de Gredos (UTM-30TUK064585) is a glacial lagoon located at 1935 m.a.s.l. (1°35´20´´ E, 40°15´15´´ N) at the Central Range (Spain). The catchment area (ca. 3.25 km^2) is mainly rocky with small grassland of *Nardus stricta* or psychroxerophytic meadows. The surface of the lagoon is 63,076 m^2. The water volume is 145,837 m^3 [22]. The renovation rate is high, and the mean annual residence time of water ranges from 18 to 24 days, depending on the annual rainfall. The lagoon has two basins separated by a rocky area with a small island. The maximum depth in the south basin is 4 m, and 6.7 m that in the north basin (Fig. 1). The surface of the lagoon remains frozen 4 to 6 months per year. Physicochemical characteristics of water are those expected for a high-mountain siliceous catchment with scarce vegetation. Laguna Grande is a typical oligotrophic aquatic ecosystem, with poorly-mineralized water, conductivities below 20 μS/cm, and low alkalinity values (0.05–0.4 meq/l) [22].

Laguna Cimera is a nearby lagoon (UTM-30TUK040596), located at 2140 m.a.s.l., 9.4 max. depth, 0.756 km^2 catchment area, 44,900 m^2 surface, and 216,890 m^3 volume.

Sample processing In December 1997, we took two sediment cores (GGE1/97 11 cm length, GGE2/97 10 cm length) from the deepest point of the Laguna Grande south basin by using a Kajak core. GGE2/97 was the core used to test the ultrasonic dispersion method (see below), and GGE1/97 was used to count the number of bacteria (see below). Cores were transported refrigerated to the laboratory and stored in upright position with water on top at 4°C. They were extruded in 2 mm intervals for the top 0–5 cm and every 5 mm below 5 cm. Subsamples were taken by cutting the sediment with sterile

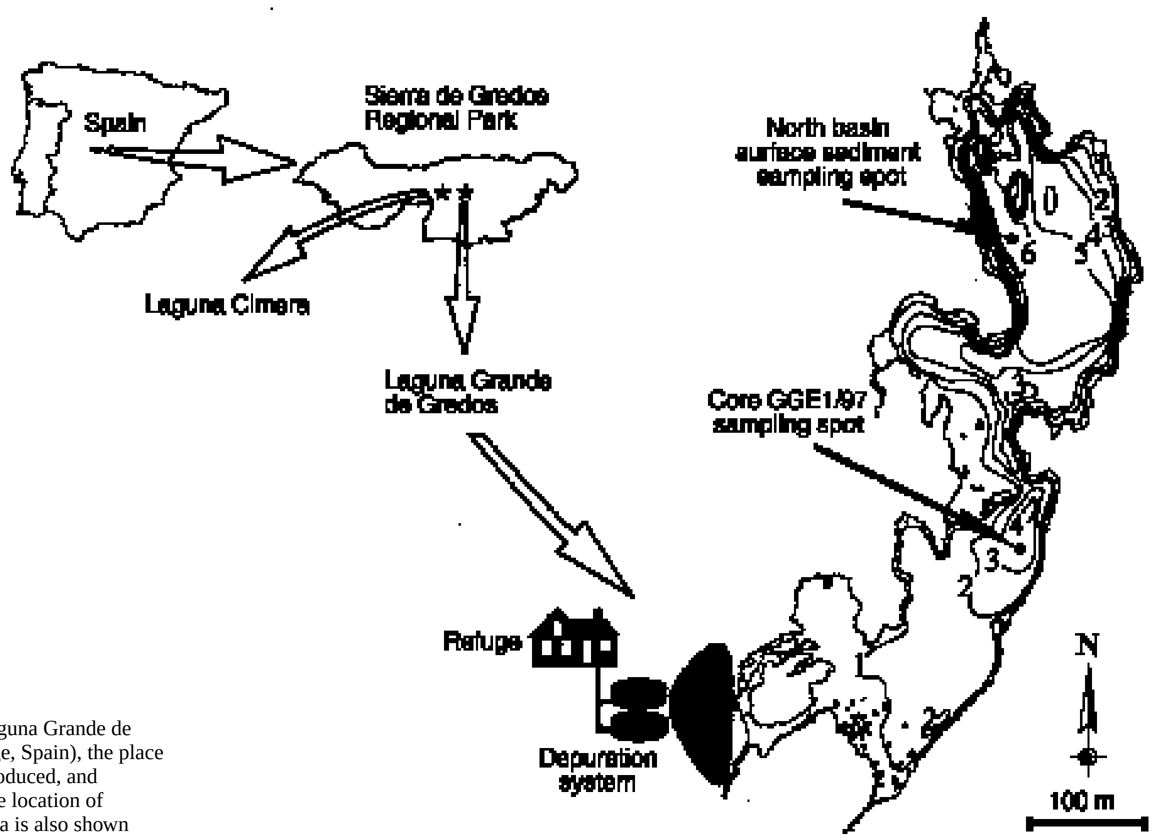

Fig. 1 Location of Laguna Grande de Gredos (Central Range, Spain), the place where sewage was produced, and sampling stations. The location of nearby Laguna Cimera is also shown

PVC sheets. To prevent contamination of samples by downcore smearing, we used the center of each level to enumerate bacteria. Remainder sediment of each interval was dried (105°C, 24 h), weighed and incinerated (550°C, 2 h) to measure fresh weight, dry weight (DW) and lost on ignition (LOI).

Subsamples were placed in distilled water and mixed with an ultrasonic bath (3 min, 40 kHz) to crush sediment aggregates. Then a magnetic stirrer was used 10 min for standardizing the mixing. Some authors [15] consider these dispersion steps to be of great importance, because endospores tend to be aggregated to various sediment particles. Sediment samples were heated at 85°C for 5 min before cultivation. Clostridia were counted by a standard dilution bank and plating, and expressed as colony-forming units per gram [CFU/g] of dry weight of sediment. Apropriate dilutions to give plates with ca. 15–450 colonies were filtered through 47 mm-diameter filters with a 0.45 µm pore size (HAW604753, Millipore) [7]. Filters were placed on Petri dishes and selective medium (iron sulfite agar [ADSA Micro 1–328]) was spread over. The plates were incubated at 37°C for 48 h in anaerobic conditions to grow sulfite-reducing clostridia. Black colonies appeared and were counted according to the plate count technique. The numbers of clostridia CFU/g were corrected by using values of dry weight in each level.

Results and Discussion

Figure 2 shows the results of sediment dry weight from core GGE1/97 (Fig. 2B), linked to the results (Fig. 2A) of a previous study of the same lake [21]. This graph relates the sediment depth to the number of sulfite-reducing clostridia CFU/g recovered per gram of sediment dry weight (Fig. 2C). The GGE1/97 core chronology was calculated by cross-correlation of dry weight percentage (% DW) of the previously ^{210}Pb dated core, taken in 1991 [21]. Figure 2A and 2B shows a good correlation between both profiles. We have considered that recovered colonies are as old as the sediment where they are found.

The graph shows an increase in the number of sulfite-reducing clostridia CFU/g (Fig. 2C) at the top of the core.

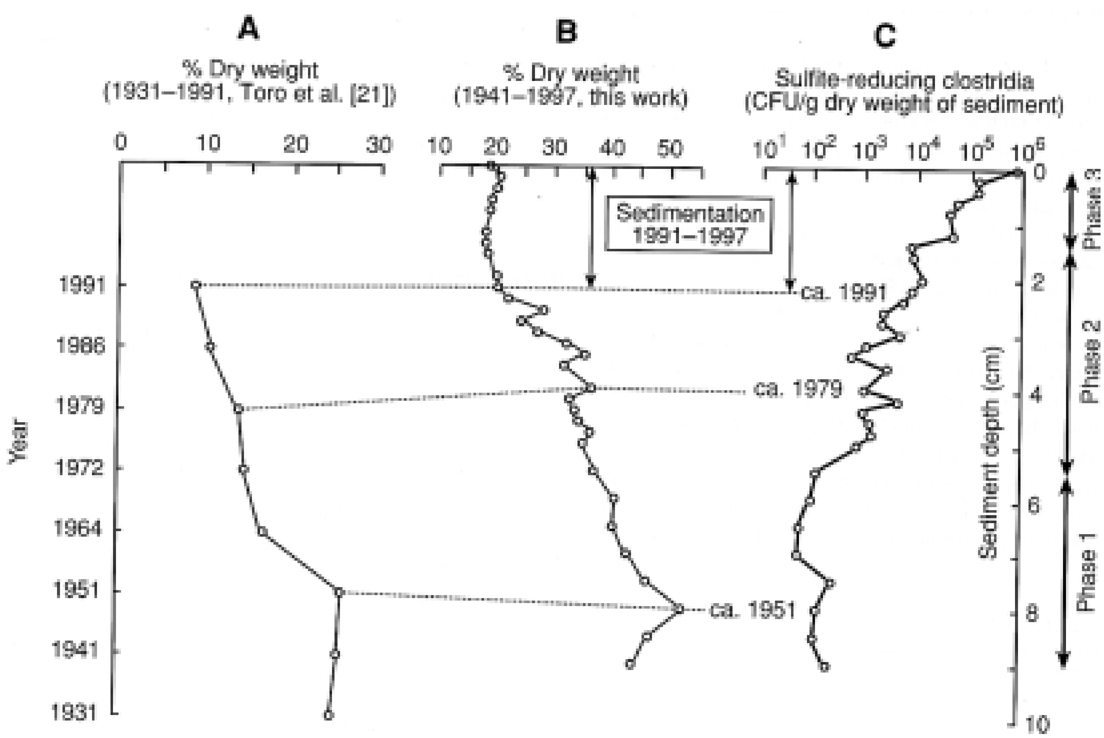

Fig. 2 Numbers of sulfite-reducing clostridia obtained from core GGE1/97 (this work), from Laguna Grande de Gredos. (A and B) Percent dry weight cross-correlation used to date GGE1/97. ^{210}Pb datation was used in the core taken in 1991 [21]. (C) Sulfite-reducing clostridia (CFU per gram of sediment dry weight) found in each level of core GGE1/97

Three deposition phases can be differentiated in the sediment: (i) From the early 1940s until the early 1970s, during which the number of sulfite-reducing clostridia CFU/g hardly changed and never surpassed 10^3; (ii) from the early 1970s until the mid 1990s, with great fluctuations, but with an upward trend of increasing numbers; and (iii) from 1995 until 1998, during which clostridia CFU/g increased from 10^3 to almost 10^6.

The general trend of increasing numbers of clostridia spores (expressed as CFU/g) since the early 1970s has an even more dramatic increase during the 1990s. During the first phase, the refuge did not exist, and only a few visitors reached the lake. Improving the accessibility to the lagoon and setting up the services provided by the refuge brought about tourism impact. This impact is reflected in the increasing numbers of clostridia CFU/g at phase two caused by a diffuse disposal of sewage from the waste treatment pond. When the depuration system started operating in 1995, pollution concentrated in the purification tanks. The closeness of these tanks to the inlet of the lake and their wrong operation produced direct leakage into the lake, which accounts for the increased numbers of clostridia CFU/g in the middle of the third phase. The highest increase corresponds to the upper millimeters, which is the sediment of the last two years. The volume of wastewater without depuration treatment during the repairs in the purification system (summer 1997) may be related to the high number of clostridia CFU/g found in the upper strata of the core. Similar patterns were observed in other paleolimnological variables [Granados & Toro, personal communication].

The same methodology was used for the cultivation of the upper layers of sediment from the north basin (top 2 mm). Table 1 shows a great difference between the clostridia CFU/g found in the upper layers of sediment from the two basins (counts from south basin is two orders of magnitude higher than those from north basin). This can be explained because the purification tanks were located nearer to the south basin. Besides, the blackwaters were wasted in summer when the water level reached its annual minimum and the entry creek was almost dry. The communication between the two basins was also reduced due to poor water interchange, and, therefore, clostridia spores settled mostly in the south basin.

Table 1 Numbers of sulfite-reducing clostridia (CFU/g) recovered from sediments of two nearby high-mountain lagoons, Laguna Grande de Gredos and Laguna Cimera (Central Range, Spain)

Site	Number of sulfite-reducing clostridia (CFU/g dry weight of sediment)
Laguna Grande south basin (upper sediment)	731×10^3
Laguna Grande north basin (upper sediment)	1392 (SD = 504, n = 3)
Laguna Cimera(upper sediment)	1234 (SD = 556, n = 10)
Laguna Cimera(10 cm depth)	194 (SD = 119, n = 7)

We also analyzed two sediment levels (0–0.2 and 10–10.2 cm depth) of Laguna Cimera, a nearby lagoon similar to Laguna Grande. We found lower counts of sulfite-reducing clostridia CFU/g in the deepest level (Table 1). The counts at the upper level were two orders of magnitude lower than those reported from the top layers of the sediment from Laguna Grande south basin. Those results agreed with a lower tourism impact and the absence of a direct disposal of sewage.

The fewer spores found at the deepest layers of the sediment might be attributed to the death of spores more than to an increase in bacterial numbers at the top layers. However, bacterial spores can survive for thousands of years with death rates of 0.0013–0.0025 per year [18]. The interval of time that we studied (38 years) allowed us to reject any possibility of exponential death with depth in the sediment, and to state that the numbers of the spores that we found were those actually settled. Thus, the numbers of sulfite-reducing clostridia in the sediment of Laguna Grande de Gredos is useful as a conservative indicator of fecal contamination from sewage disposal, and can be used to assess pollution in aquatic environments of high-mountain areas. Our results supports other studies that propose clostridia as a good indicator of past human pollution.

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